# A Circuit Mechanism Underlying Suppression of Circadian Signals by Homeostatic Sleep Drive

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## Abstract

Sleep is controlled by homeostatic mechanisms, which drive sleep following periods of wakefulness, and a circadian clock, which regulates sleep timing in a daily cycle. Homeostatic sleep drive sometimes overrides the clock, such that recovery sleep after deprivation occurs outside the normal circadian rest period. However, mechanisms underlying this effect are unknown. We find that sleep-promoting dorsal fan-shaped body (dFB) neurons, effectors of homeostatic sleep in *Drosophila*, are presynaptic to *hugin*+ neurons, previously identified as circadian output neurons regulating locomotor activity rhythms. Sleep deprivation decreases *hugin*+ neuronal activity, which likely suppresses circadian control to promote recovery sleep driven by dFB neurons. Indeed, removal of *hugin*+ neurons increases sleep-promoting effects of dFB neurons. Trans-synaptic mapping reveals that *hugin*+ neurons feed back onto s-LNv central clock neurons, which also show Hugin-dependent decreased activity upon sleep loss. These findings identify a circuit-based mechanism through which sleep drive modulates the circadian system to promote recovery sleep following deprivation.

## Introduction

Sleep is regulated by two processes, circadian and homeostatic (Borbély, Daan, Wirz-Justice, & Deboer, 2016). The endogenous circadian clock, together with its downstream pathways, is synchronized to external day-night cycles and determines the timing of sleep to generate 24-hour rhythms in sleep and wake. The homeostatic process tracks sleep:wake history and generates sleep drive based on the extent of wakefulness. Sleep homeostasis can be overtly seen as an increase in sleep duration and depth after prolonged wakefulness. Generally, circadian and homeostatic processes are studied as separate mechanisms that regulate sleep, although they clearly intersect and in a daily cycle the two processes are coordinated to determine the onset of sleep at night. Importantly, the homeostatic system can sometimes over-ride circadian signals to drive sleep at the *wrong* time of day, for instance in the morning following a night of sleep deprivation. However, the neuronal mechanisms by which circadian and homeostatic pathways signal to each other are unknown.

The functions and regulation of sleep are extensively studied in model organisms, such as *Drosophila melanogaster* (Dubowy & Sehgal, 2017). In the *Drosophila* brain, the circadian clock is expressed in ~150 clock neurons that are organized into neuroanatomical groups, of which the ventral and dorsal lateral neurons are the most important for driving rhythms of locomotor activity (Delventhal et al., 2019; Schlichting, Díaz, Xin, & Rosbash, 2019). The small ventrolateral neurons (s-LNvs) link to other brain regions is through different circuits. One of those circuits connects the sLNvs to the site of the motor ganglion, the thoracic nerve cord: s-LNvs → DN1s → *Dh44*+ neurons → *hugin*+ neurons→ ventral nerve cord (King & Sehgal, 2020). *Dh44*-expressing neurons in the pars intercerebralis regulate locomotor activity rhythms in part through the signaling of DH44 neuropeptide to *hugin*-expressing neurons in the subesophageal zone (SEZ) (Cavanaugh et al., 2014; King et al., 2017). *Dh44*+ and *hugin*+ circadian output neurons do not contain canonical molecular clocks, but display cycling in

neuronal activity or peptide release, likely under control of upstream circadian signals (Bai et al., 2018; Cavey, Collins, Bertet, & Blau, 2016; King et al., 2017). However, links between these neurons and loci regulating sleep homeostasis have not been identified yet.

Regulation of sleep homeostasis in flies involves the central complex and mushroom body (Donlea, 2017; Joiner, Crocker, White, & Sehgal, 2006; Pitman, McGill, Keegan, & Allada, 2006; Sitaraman et al., 2015). Recent studies have focused on a group of sleep-promoting neurons that project to the dorsal fan-shaped body (dFB) in the central complex. Activation of dFB neurons promotes sleep (Donlea, Thimgan, Suzuki, Gottschalk, & Shaw, 2011; Ueno et al., 2012), and these neurons are required for normal sleep rebound after deprivation (Qian et al., 2017). dFB neurons receive input signals from R2 ellipsoid body neurons, which track sleep need (Liu, Liu, Tabuchi, & Wu, 2016), but whether the dFB neurons signal to circadian neurons remains to be elucidated.

To determine how the two sleep-regulating processes interact, we explored possible connections between the relevant circuits. We find that sleep-promoting dFB neurons are presynaptic to *hugin*+ circadian output neurons. *hugin*+ neurons are dispensable for determining daily sleep amount, but they appear to counter sleep-promoting effects of dFB neurons, such that ablation of *hugin*+ neurons enhances sleep driven by the dFB. Also, activation of *hugin*+ neurons reduces recovery sleep after nighttime sleep loss, and Hugin peptide mutations enhance sleep rebound after sleep deprivation. We find that *hugin*+ neurons target PDF+ s-LNv clock neurons, and both circadian neuronal groups show decreases in intracellular $Ca^{2+}$ levels following sleep deprivation. Based on these new findings, we propose a circuit mechanism by which a sleep homeostatic circuit downregulates wake-promoting outputs of the circadian clock to allow sleep at times when the circadian system typically promotes wake.

## Results

### Sleep-promoting dFB neurons are presynaptic to *hugin*+ circadian output neurons

We previously found that *hugin*+ neurons in the subesophageal zone (SEZ) project back to the region of DH44+ neurons (King et al., 2017) and even beyond it into the superior medial protocerebrum (SMP). As some SMP neurons project to the dFB, we sought to determine if *hugin*+ neurons contact the dFB. We hypothesized that the *hugin*+ neurons signal to the dFB to control the timing of sleep and so speculated that post-synaptic sites of the dFB would be in the SMP. Indeed, using the sleep-promoting 23E10-Gal4 driver (Donlea, Pimentel, & Miesenböck, 2014; Donlea et al., 2018; Pimentel et al., 2016; Qian et al., 2017) to label dFB neuron dendrites and simultaneously using the LexA system to mark *hugin*+ neuron pre-synaptic sites, and found that both sets of projections localize to the SMP (Supplemental Figure 1). However, published images also suggest presynaptic sites of dFB neurons in the SMP, even though these are primarily localized in a single dorsal layer of the fan-shaped body (Cavanaugh, Vigderman, Dean, Garbe, & Sehgal, 2016; Donlea et al., 2018; Li et al., 2009; Ni et al., 2019). Using *23E10-LexA* to express *Rab3::GFP* we confirmed the presence of presynaptic projections of *23E10*+ neurons in the dFB and SMP, albeit with a weaker signal in the SMP (Figure 1A). Additionally, we expressed *brp-short* $^{GFP}$, a nonfunctional 754-residue portion of bruchpilot (BRP) that localizes to presynaptic active zones (Fouquet et al., 2009; Schmid et al., 2008), in *23E10*+ neurons. This pre-synaptic marker labeled projections in both the dFB and SMP (Figure 1B).

To look for a possible synaptic connection between *23E10*+ and *hugin*+ neurons, we used a trans-synaptic GFP fluorescence reconstitution assay (nSyb-GRASP). This system uses the expression of a split GFP, one part tethered to neuronal Synaptobrevin (nSyb::spGFP1-10) in the putative presynaptic cells and the complement tethered to the membrane (CD4::spGFP11) of the putative postsynaptic neurons (Macpherson et al., 2015). Split GFP

fragments only reconstitute at close membrane contacts, which are identified by GFP fluorescence (Feinberg et al., 2008). Since nSyb is trafficked to the presynaptic vesicle membrane, nSyb-GRASP identifies membrane contacts specifically at synapses. We first tested the nSyb-GRASP tool by co-expressing presynaptic nSyb::spGFP1-10 and complementary CD4::spGFP11 in *23E10*+ dFB neurons. In these flies, GFP reconstituted in both the dFB and SMP (Figure 1C left), confirming that *23E10*+ dFB neurons have presynaptic sites in both these regions. In flies with the presynaptic nSyb::spGFP1-10 expressed in *23E10*+ dFB neurons and complementary CD4::spGFP11 expressed in *hugin*+ neurons, fluorescent GFP reconstituted in the SMP but not in the dFB, or in the SEZ (Figure 1C middle, top and bottom). We also performed the reciprocal experiment, with nSyb::spGFP1-10 expressed in the *hugin*+ neurons and complementary CD4::spGFP11 expressed in *23E10*+ dFB neurons, but did not observe any GFP fluorescence in the brain (Figure 1C right). Additionally, no GFP fluorescence was also observed in brains expressing either half of the GRASP components and imaged under the same conditions (data not shown). These results suggest that, contrary to our original hypothesis that *hugin*+ neurons project to the dFB, *23E10*+ dFB neurons are presynaptic to *hugin*+ neurons in the SMP.

**Disrupting the activity of *hugin*+ neurons is not sufficient to alter sleep amount or recovery sleep**

The connection between *23E10*+ dFB and *hugin*+ neurons led to the question of whether *hugin*+ neurons also regulate sleep. To test this, we expressed temperature-sensitive *TrpA1* channel (Pulver, Pashkovski, Hornstein, Garrity, & Griffith, 2009) in *hugin*+ neurons and activated them with high temperature while measuring sleep behavior. In other experiments, we expressed temperature-sensitive *shibire*$^{ts}$, a dominant-negative dynamin gene (Kitamoto, 2001), to inhibit synaptic transmission from *hugin*+ neurons at high temperature. As previously reported

(Donlea et al., 2011; Ni et al., 2019; Ueno et al., 2012), activation of *23E10*+ dFB neurons at high temperature with *TrpA1* led to increased sleep (data not shown). However, we did not observe changes in sleep amount when *hugin*+ neurons were activated with *TrpA1* (Supplementary Figure 2A) or inhibited with *shibire*$^{ts}$ (Supplementary Figure 2B). While there were no changes to sleep, *hugin*>*shibire*$^{ts}$ flies were less active than control flies, as measured by number of beam crossings per day (Supplemental Figure 2C), which confirms our previous findings that *hugin*+ neurons regulate locomotor activity (King et al., 2017).

Since mechanisms that participate in baseline and sleep recovery may be different, we asked whether *hugin*+ neurons play a role in regulating sleep homeostasis. We used the same thermogenetic approaches to activate or inhibit the *hugin*+ neurons while simultaneously sleep depriving the flies at night using a mechanical method. After sleep deprivation, recovery sleep was monitored in the flies during the daytime. We found no significant difference in recovery sleep between the experimental and control genotypes when *hugin*+ neurons were activated or inhibited (Supplemental Figure 2D). As mechanical sleep deprivation can recruit multiple pathways to elicit rebound (Dubowy et al., 2016), it is possible that disrupting the activity of *hugin*+ neurons alone does not affect sleep amount or homeostasis.

**Sleep deprivation decreases $Ca^{2+}$ levels in *hugin*+ neurons**

Although *hugin*+ neurons did not affect sleep, we considered the possibility that they were affected by sleep loss. Sleep is correlated with changes in neuronal activity in sleep-regulatory circuits, including the MB, dFB, and R2 ellipsoid body (Bushey, Tononi, & Cirelli, 2015; Liu et al., 2016; Sitaraman et al., 2015; Yap et al., 2017). For example, sleep-promoting dFB neurons tend to be more electrically active after sleep deprivation, when sleep pressure is high, than in rested flies (Donlea et al., 2014). If the *hugin*+ neurons receive signals from sleep-

promoting dFB neurons, activity of *hugin*+ neurons may change with sleep pressure. To test this, we measured intracellular $Ca^{2+}$ levels as a readout of neuronal activity in *hugin*+ neurons using CaLexA (Calcium-dependent nuclear import of LexA) (Masuyama, Zhang, Rao, & Wang, 2012). The CaLexA system drives expression of GFP in response to sustained increases in intracellular $Ca^{2+}$ levels. We used *hugin-GAL4* to express CaLexA-GFP transgenes and *UAS-CD8:RFP* for normalizing the GFP signal. We deprived *hugin>CaLexA-GFP;RFP* flies of sleep for nine hours at the end of the night (ZT 15-24) and subsequently collected flies for CaLexA measurements (Figure 2A). A control group, flies that were not sleep deprived, was assayed at the same time of day as the deprived group. In the sleep-deprived flies, CaLexA-dependent GFP signal intensity in *hugin*+ neurons was lower than that in control flies (Figure 2B-C). To rule out a general effect of sleep deprivation on $Ca^{2+}$, we also tested if sleep deprivation affects $Ca^{2+}$ levels in *Dh44*+ neurons, another group of circadian output neurons (Cavanaugh et al., 2014). However, the CaLexA-GFP signal in *Dh44*+ neurons was not significantly different between the sleep-deprived and control flies (Figure 2D). Thus, sleep deprivation specifically affects $Ca^{2+}$ levels of *hugin*+ neurons, suggesting that the homeostat engages *hugin*+ neurons.

### ***hugin*+ neurons are effectors of 23E10+ sleep-promoting dFB neurons**

If *hugin*+ neurons are downstream of *23E10*+ sleep-promoting dFB neurons, they could affect the sleep-promoting output of *23E10*+ neurons. To test this hypothesis, we activated *23E10*+ neurons in flies where *hugin*+ neurons were either ablated (Figure 3A) or simultaneously activated (Figure 3B). We used the GAL4/UAS system to express the proapoptotic gene, *reaper*, to genetically ablate *hugin*+ neurons, and used the LexA/LexAop system to express 2 copies of *TrpA1* to activate *23E10*+ dFB neurons. Thermogenetic activation of the *23E10*+ neurons using the LexA/LexAop system (*23E10-LexA>LexAop-TrpA1(2x); +>UAS-reaper*) led to sleep increase during the day as reported previously (Donlea et al., 2011;

Ueno et al., 2012) (Figure 3A). The sleep increase was not as large as that observed in *23E10-GAL4>UAS-TrpA1* flies (Figure 3B), likely because 23E10-LexA is less effective than 23E10-GAL4 as a transcriptional activator. Activation of *23E10+* neurons in flies lacking *hugin+* neurons (*23E10-LexA>LexAop-TrpA1(2x); hugin-GAL4>UAS-reaper*) enhanced the typical daytime increase in sleep (Figure 3A, second graph). This result is consistent with *hugin+* neurons inhibiting the output of *23E10+* neurons. When the *23E10+* and *hugin+* neurons were simultaneously activated using the GAL4/UAS system, there was no change to the sleep-promoting effects of *23E10+* dFB neurons (Figure 3B). This could reflect suppression of even the increased *hugin+* neuron activity by *23E10+* neurons or, alternatively, signaling through other *23E10+* dFB targets such as the helicon cells (Donlea et al., 2018).

In the thermogenetic sleep experiments, we also observed significant heat-induced sleep loss during the night in control animals (Figure 3A, third graph). This is consistent with the effect of high temperature, which reorganizes sleep such that daytime sleep increases and night-time sleep decreases. Indeed, effects of dFB activation on night-time sleep are often manifested as unchanged sleep levels relative to decrease seen in controls. The heat-induced nighttime sleep loss also engages the homeostat resulting in sleep increase the next day (Parisky, Agosto Rivera, Donelson, Kotecha, & Griffith, 2016). To determine if *hugin+* neurons affect recovery sleep after heat-induced nighttime sleep loss, we maintained flies for a day at 31°C (high temperature), after which they were returned to 21°C (low temperature) to recover sleep. Recovery sleep was determined by comparing sleep on recovery day with sleep at baseline day, both days at 21°C. Ablation of *hugin+* neurons did not affect the amount of sleep loss at 31°C or the amount of recovery sleep at 21°C after heat-induced sleep loss (Figure 3A). Thermogenetic activation of *hugin+* neurons also did not affect the amount of sleep loss at 31°C, when compared to the controls (Figure 3B, third graph). However, after return to 21°C, recovery sleep was decreased during the 24hr period following sleep loss in flies where *hugin+*

cells were activated with *TrpA1,* compared to the control groups or flies with *23E10>TrpA1* activation (Figure 3B).

Despite having more sleep than controls during the high temperature, flies with activated *23E10*+ dFB neurons recovered additional sleep after the transition back to low temperature. However, flies subjected to simultaneous activation of *23E10*+ and *hugin*+ neurons showed decreased sleep recovery at 21°C, similar to that seen with *hugin>TrpA1* activation alone (Figure 3B). We hypothesize that homeostatic pressure triggered by heat-induced sleep loss inhibits the activity of *hugin*+ circadian neurons to promote recovery sleep. Activation of *hugin*+ neurons reduces this recovery.

To verify that heat-induced sleep loss affects the activity of *hugin*+ neurons, we used the CaLexA system to measure $Ca^{2+}$ in *hugin>CaLexA-GFP;RFP* flies after a full day at 30°C. A control group, flies that were kept at a constant low temperature, was assayed at the same time of day as the heat-exposed group. Compared to the baseline night at 21°C, *hugin>CaLexA-GFP;RFP* flies ($n$ = 16) lost an average 208.13 minutes of sleep (sd = 79.10) during the night at 30°C (Supplemental Figure 3A). The heat-induced sleep loss was accompanied with decreased CaLexA-GFP signal in *hugin*+ neurons compared to the control (Supplemental Figure 3B), showing that sleep loss due to heat also inhibits the activity of *hugin*+ neurons.

To determine if the ability of *hugin*+ neurons to affect the sleep homeostat is dependent on Hugin peptide signaling, we generated *hugin* mutants. We used CRISPR-CAS9 system to produce mutant alleles of *hugin* that affect expression of one or both of the encoded neuropeptides, Hugin-γ and Pyrokinin 2 (PK2), encoded by the *hugin* locus (Melcher, Bader, Walther, Simakov, & Pankratz, 2006). The $hugin^{PK}$ mutant contains a 1-base pair deletion that truncates the PK2 peptide, while the $hugin^{\Delta Ex3}$ mutant lacks the majority of exon 3 and is predicted to eliminate both neuropeptides. The $hugin^{PK2}$ mutants show weaker circadian rhythms, while the $hugin^{\Delta Ex3}$ flies show a small lengthening of circadian period (Supplemental

Figure 4B); thus, period effects may arise from the Hugin peptide, and we suggest that the stronger rhythms in the null allele reflect some developmental compensation. Interestingly, both mutants exhibit decreases in baseline sleep (Supplemental Figure 4A). Since our data support the idea that *hugin*+ neurons are effectors of the sleep homeostat, we measured rebound sleep in both mutants after mechanical deprivation of sleep for nine hours at the end of the night (ZT 15-24). We found that both mutants exhibited increased rebound sleep (Figure 3C) indicating that effects of *hugin*+ neurons on recovery sleep are mediated through peptides made by the *hugin* locus.

### ***Pdf*+ clock neurons are targets of *hugin*+ neurons**

We previously showed that *hugin*+ neurons project into the ventral nerve cord (King et al, 2017). To identify additional targets of these neurons, we used *trans*-Tango, a pan-neuronal trans-synaptic labeling system (Talay et al., 2017) in which a tethered ligand is expressed at the synapses of genetically defined neurons and postsynaptic partners are identified through activation of a synthetic signaling pathway, visualized as expression of fluorescent tdTomato. Presynaptic neurons are simultaneously labeled with myr::GFP, a different fluorescent protein. We expressed the *trans*-Tango ligand in *hugin*+ neurons and observed *trans*-Tango-dependent signal in many brain regions, including the pars intercerebralis, mushroom body lobes, mushroom body calyx and pedunculus, SMP, subesophageal zone, and accessory medulla (Figure 4A top). In addition, we found that *hugin*+ neurons project into the accessory medulla (Figure 4A top right, magenta, arrowheads). The postsynaptic neurons in the accessory medulla were reminiscent of small ventrolateral neurons (s-LNvs), prompting us to label for PDF peptide and confirm that a subset of the postsynaptic partners observed in *hugin>trans-Tango* flies is PDF-positive. *Pdf*+ neurons are subdivided into the small (s-LNv) and large (l-LNv) ventrolateral neurons, each group containing 4-5 neurons per hemisphere (Helfrich-Förster et al., 2007). The

*trans*-Tango-dependent signal was more intense in the s-LNvs than in the l-LNvs (Figure 4A bottom), indicating that s-LNvs are primary targets of *hugin*+ neurons.

Our data demonstrate a circuit that links sleep homeostasis centers to circadian clock neurons (*23E10*+ dFB → *hugin*+ SEZ → *Pdf*+ s-LNvs), and suggest a potential mechanism for homeostatic components to regulate circadian clock outputs. To test whether the activity of *Pdf*+ neurons themselves is altered with sleep deprivation, we used CaLexA to measure $Ca^{2+}$ level changes in *Pdf*+ neurons during sleep deprivation. With mechanical sleep deprivation, the CaLexA-GFP signal in both *Pdf*+ s-LNv and l-LNv cell bodies was lower in the sleep-deprived flies as compared to controls (Figure 4B,C). We next tested if the $Ca^{2+}$ decrease in *Pdf*+ neurons after deprivation is dependent on Hugin peptide signaling. By comparing the activity of *Pdf*+ neurons in both control and sleep deprivation conditions, we found that in s-LNvs, sleep deprivation resulted in a decrease in $Ca^{2+}$ levels in the control $hugin^{\Delta Ex3/+}$ group, but not in $hugin^{\Delta Ex3/\Delta Ex3}$ mutants (Figure 5A,B). This result is of particular interest since the *trans*-Tango experiment suggests that s-LNvs are primary targets of *hugin*+ neurons. Notably, sleep deprivation did not affect $Ca^{2+}$ levels in l-LNvs $hugin^{\Delta Ex3/\Delta Ex3}$ groups compared to $hugin^{\Delta Ex3/+}$ controls. Thus, sleep deprivation acts through Hugin peptides to suppress the activity of s-LNvs.

## Discussion

The circadian clock and homeostat both regulate sleep, but it is not clear how the two processes functionally interact. We identify a circuit-based mechanism in the fly brain that links output arms of the sleep homeostat and the circadian clock. Homeostatic sleep drive signals through *hugin*+ neurons to suppress circadian outputs, thereby, allowing for sleep at times when the circadian system typically promotes wake. We also find that *hugin*+ circadian output

neurons feedback to s-LNvs, the central clock neurons. Thus, the sleep homeostat influences outputs of the circadian clock by modulating the activity of circadian output neurons and clock neurons (Figure 5C).

Our data indicate that a sleep homeostat effector, *23E10*+ dFB neurons, project to *hugin*+ circadian output neurons. Previously, we showed that a circuit from s-LNvs → DN1 → *Dh44*+ neurons → *hugin*+ neurons controls locomotor activity rhythms. *hugin*+ neurons are locomotor activity-promoting, especially during the evening (day-to-night transition) peak of activity (Cavanaugh et al., 2014; King et al., 2017). We suggest that *23E10*+ sleep-promoting dFB neurons directly or indirectly exert a negative effect on *hugin*+ activity-promoting neurons. First, the sleep-promoting effect of *23E10*+ dFB neurons is enhanced during the daytime when the *hugin*+ neurons are removed. Second, neuronal activity of *hugin*+ neurons is suppressed with sleep deprivation, while dFB neurons become more active after sleep deprivation (Donlea et al., 2014). In addition, we find that activating *hugin*+ neurons during a period of heat-induced sleep loss leads to less recovery sleep. Activation of *hugin*+ neurons during mechanical sleep deprivation does not affect rebound, likely because mechanical deprivation recruits multiple pathways (Dubowy et al., 2016). We note also that in the mechanical sleep deprivation experiment, *hugin*+ neurons were activated prior to deprivation, while heat-induced sleep loss was concurrent with their activation; it is possible that prior activation resets the homeostatic system to a different set point of neural activity. Importantly, we also report increased recovery sleep in *hugin* mutants following deprivation, indicating that the homeostatic sleep-suppressing role of *hugin*+ neurons is mediated by the Hugin peptide. Our data suggest that during sleep deprivation, the homeostat not only generates sleep drive but also actively disengages activity-promoting circuits.

The circadian clock can regulate sleep by cell-intrinsically controlling the neuronal activity of clock neurons, such as the LNvs. The wake-promoting effect of the LNvs is light-dependent and largely comes from the l-LNv subset (Parisky et al., 2008; Shang, Griffith, &

Rosbash, 2008; Sheeba, Fogle, et al., 2008). While s-LNvs alone are not sufficient to promote wake, downregulation of PDF receptor in s-LNvs increases sleep, suggesting PDF signaling to s-LNvs modulates wake-promoting effects of l-LNvs (Parisky et al., 2008; Shang et al., 2008). In addition, the downregulation of short Neuropeptide F signaling between s-LNvs and l-LNvs decreases nighttime sleep (Shang et al., 2013). Notably, both s-LNvs and l-LNvs show more depolarized resting membrane potentials during the day than during the night, supporting the idea that LNvs are more active during times of increased arousal (Cao & Nitabach, 2008; Sheeba, Gu, Sharma, O'Dowd, & Holmes, 2008). A recent study demonstrated that another subset of clock neurons, $LPN^{AstA}$ neurons, project to the dFB to promote sleep and could serve as a mechanism of circadian control of the sleep homeostat (Ni et al., 2019).

Our work suggests that sleep homeostasis also influences the neuronal activity of the LNvs. It was previously reported that sleep loss due to social enrichment is associated with an increased number of synapses in the LNv projections into the medulla, a brain region that processes visual information from the eyes (Donlea, Ramanan, & Shaw, 2009). Here, we report $Ca^{2+}$ levels in LNvs decrease with sleep deprivation, which we hypothesize dampens the wake-promoting effects of LNvs to allow for recovery sleep. It is possible that decreased $Ca^{2+}$ levels in LNvs with sleep deprivation precedes synaptic downscaling that is thought to occur with sleep recovery (Donlea et al., 2009). *hugin* mutants do not exhibit a decrease in s-LNv Ca2+ levels with sleep deprivation, which suggests that Hugin peptide signaling is involved in the suppression of s-LNv activity with sleep deprivation. While we have only mapped a connection from *23E10*+ dFB to the LNv wake-promoting clock neurons through *hugin*+ neurons, it is likely that other sleep homeostat pathways also modulate LNvs. Notably, GABA and myoinhibitory peptide signal to LNvs to regulate sleep, although the source of these neuromodulators is not known yet (Chung, Kilman, Keath, Pitman, & Allada, 2009; Oh et al., 2014; Parisky et al., 2008).

The mammalian orthologue of Hugin, Neuromedian U (NMU) (Melcher et al., 2006), has been previously implicated in sleep regulation. While NMU injection in rats does not change

total sleep time, it changes sleep architecture (Ahnaou & Drinkenburg, 2011). NMU overexpression decreases sleep in zebrafish (Chiu et al., 2016), which is in contrast to our finding that *hugin* CRISPR mutants exhibit decreased baseline sleep. Chiu et al did not report alterations in baseline sleep levels in their NMU knockout fish. While neither of these studies evaluated the potential role of NMU in homeostatic sleep rebound following deprivation, NMU overexpression increases stimulus-evoked arousal duration in zebrafish during sleep. To the extent that increased arousal reflects decreased sleep drive, this would be consistent with our finding that *hugin*+ neuron activation during sleep deprivation suppressed rebound sleep.

In *Drosophila,* influences of sleep homeostatic mechanisms on the circadian system have not been demonstrated. In rodents, sleep deprivation dampens electrical activity in the suprachiasmatic nucleus (SCN) for up to 7 hours and reduces the ability of the circadian clock to phase shift by light (Borbély et al., 2016; Challet, Turek, Laute, & Van Reeth, 2001; Deboer, Détári, & Meijer, 2007; Mistlberger, Landry, & Marchant, 1997). We find a similar effect in flies, where neuronal activity is depressed in LNv central clock neurons and remained depressed even 5 hours after the deprivation ended (data not shown). In the rodent model, the mechanism mediating the reduced SCN activity is not clear, but may involve serotonin signaling from the raphe dorsalis (Deboer, 2018). We show here that effects of sleep deprivation on LNvs are mediated by Hugin signaling from output neurons that are targets of a major sleep homeostatic locus. Importantly, sleep deprivation does not appear to shift rest:activity patterns in flies (Hendricks et al., 2000) and sleep deprivation does not affect the rodent SCN (Curie, Maret, Emmenegger, & Franken, 2015), suggesting that the clock is unperturbed. Therefore, sleep homeostasis appears to influence primarily clock outputs.

## Methods

*Drosophila melanogaster*

Flies were maintained on cornmeal-molasses medium. For thermogenetic and *trans*-Tango experiments, flies were raised at 18°C, and all other flies were maintained at 25°C. $w^{1118}$ iso31 strain was used as the wild type strain. For sleep behavior experiments, transgenic lines were backcrossed into the iso31 genetic background. For controls, UAS and GAL4 fly lines were tested as heterozygotes after crossing to iso31. The following flies were from the Bloomington Drosophila Stock Center: *23E10-GAL4* (#49032) (Jenett et al., 2012), *23E10-LexA* (#52693) (Pfeiffer et al., 2010), *Hugin-GAL4* (#58769) (Melcher & Pankratz, 2005), *Hugin-LexA* (#52715), *Dh44-GAL4* (#39347), *UAS-CD8::RFP* (#32219), *LexAop-Rab3::GFP* (#52239) (Shearin, Dvarishkis, Kozeluh, & Stowers, 2013), *LexAop-6xmCherry-HA* (#52271), *UAS-nSyb::GFP1-10, LexAop-CD4::GFP11* (#64314), *UAS-reaper* (#5773) (White, Tahaoglu, & Steller, 1996). *Trans*-Tango fly was a gift from G. Barnea. CaLexA fly was a gift from J.W. Wang. *UAS-TrpA1* was a gift from L.C. Griffith. *UAS-shibire*$^{ts}$ (*20XUAS-IVS-Shibire[ts1]-p10-INS*) and *LexAop-TrpA1* (chromosome 2) were gifts from G. Rubin (Pfeiffer, Truman, & Rubin, 2012). *LexAop-TrpA1* (chromosome 3) was a gift from S. Waddell (Burke et al., 2012).

Generation of *hugin* mutants

*hugin*$^{\Delta Ex3}$ and *hugin*$^{PK2}$ mutants were generated with the CRISPR-CAS9 system. The guide RNA sequences for generating *hugin*$^{\Delta Ex3}$ were 5' GGGAGCCCGCTTATCGCGTG 3' and 5' GGAGGACGGAGGACGAGCCC 3', and the guide RNAs for *hugin*$^{PK2}$ were 5' GTGCCGTTCAAGCCACGCCT 3' and 5' GGCAAACGTGCTCAAGTGTG 3'. Guide RNAs were cloned into the pCFD4 plasmid (Port, Chen, Lee, & Bullock, 2014), and the plasmids encoding the guide RNAs were injected into *vasa-Cas9* flies (BDSC # 51323) (Gratz et al., 2014) at Rainbow Transgenic Flies, Inc (Camarillo, CA). Mutations in the F1 generation were identified with PCR screening and confirmed with Sanger sequencing. *hugin*$^{\Delta Ex3}$ is a 260-bp deletion

(dm6/chr3R:12,528,601-12,528,860), and *hugin*$^{PK2}$ is a 1-bp deletion (dm6/chr3R: 12,528,750). The mutant alleles were backcrossed for five generations into iso31 background.

Immunohistochemistry

For polarity labeling and CaLexA experiments, ~7 d old females raised at 25°C were used. For *trans*-Tango experiments, ~15-20 d old females raised at 18°C were used, as previously described (Talay et al., 2017). All fly brains were dissected in phosphate-buffered saline with 0.1% Triton-X (PBST) and fixed with 4% formaldehyde in PBS for 20 min at room temperature. Brains were rinsed 3 x 10 min with PBST, blocked in 5% Normal Goat Serum in PBST (NGST) for 60 min, and incubated in primary antibody diluted in NGST for >16 h at 4°C. Brains were rinsed 3 x 10 min in PBST, incubated 2 h in secondary antibody diluted in NGST, rinsed 3 x 10 min in PBST, and mounted with Vectashield media (Vector Laboratories Inc.). Primary antibodies used were: rabbit anti-GFP at 2µg/mL (Thermo Fisher Scientific Inc. A-11122), rat anti-RFP at 1µg/mL (ChromoTek 5F8), mouse anti-BRP at 1:1000 (Developmental Studies Hybridoma Bank nc82), rat anti-HA at 1µg/mL (Roche clone 3F10), and mouse anti-PDF at 0.3µg/mL (Developmental Studies Hybridoma Bank c7-c). Secondary antibodies were from Thermo Fisher Scientific Inc. and used at 1:1000: Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-rat, Alexa Fluor 647 goat anti-rat, Alexa Fluor 647 goat anti-mouse.

nSyb-GRASP

nSyb-GRASP flies were dissected in extracellular saline (103 mM NaCl, 3 mM KCl, 1 mM $NaH_2PO_4$, 4 mM $MgCl_2$, 10 mM D-(+)-trehalose dehydrate, 10 mM D-(+)-glucose, 5 mM N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid, 26 mM $NaHCO_3$, pH 7.4). Dissected brains were exposed to a high concentration of KCl to increase GRASP signal, as previously described (Macpherson et al., 2015). Dissected brains were incubated in 1 ml 70 mM KCl in saline three times (~5 s per KCl incubation), alternating with 1 ml saline (~5 s per wash), and then transferred to 1 mL saline to incubate for 10 minutes. Brains were fixed with 4%

formaldehyde in PBS for 20 minutes at room temperature, rinsed 3 x 10 min in PBST, and mounted with Vectashield media. Endogenous GRASP signal without antibody labeling was imaged.

Confocal Microscopy

Eight-bit images were acquired using a Leica TCS SP5 laser scanning confocal microscope with a 40x/1.3 NA or 20x/0.7 NA objective and a 1-μm z-step size. Maximum intensity z-projection images were generated in Fiji, a distribution of ImageJ software (Schindelin et al., 2012).

Sleep Behavior Assay

Individual ~7 d old female flies were loaded into glass tubes containing 5% sucrose and 2% agar. Locomotor activity was monitored with the Drosophila Activity Monitoring system (DAMS) (Trikinetics, Waltham, MA). Flies were monitored for sleep in a 12 h:12 h (12:12) light:dark cycle at 25°C for CaLexA experiments or at 21°C for thermogenetic experiments. Incubator temperature shifts occurred at lights-on, Zeitgeber time (ZT) 0. For mechanical sleep deprivation experiments, flies were loaded into the DAMS and sleep deprived during the night by shaking on an adapted vortex for 2 s randomly within every 20 s interval. Sleep was defined as 5 consecutive min of inactivity. Sleep analysis was performed with PySolo software (Gilestro & Cirelli, 2009). Data from flies that survived the duration of the experiments were pooled and analyzed. Behavioral data were analyzed with one-way analysis of variance (ANOVA) with Tukey's test as the post hoc test to compare means between groups. Differences between groups were considered significant if $P < 0.05$ by the post hoc test.

Circadian Rest:Activity Rhythms Behavior Assay

Rest:activity rhythm assays were performed and analyzed as described in King et. al. 2017. Flies were entrained to a 12 hr light: 12 hr dark (LD) cycle from birth and put in total darkness for analysis at ~7 d old. Data from days 3-9 for flies that survived the duration of the experiment

were analyzed. Period and 24hr FFT were analyzed by one-way ANOVA with Tukey's test in Prism 7 for Mac Os X.

CaLexA Analysis

Fluorescence intensity measurement was performed in Fiji. Regions of interest (ROIs) were manually drawn to encompass individual RFP-positive cell bodies, and mean pixel intensities of RFP and GFP were measured from the ROI. For each cell, the CaLexA-GFP/RFP signal (arbitrary unit, a.u.) was calculated as a ratio between the mean pixel intensities of GFP and RFP. For each brain, the average CaLexA-GFP/RFP signal of a cell is a sample point. Welch's *t*-test was used to compare differences in CaLexA-GFP/RFP signal between sleep-deprived and control groups. The two-way ANOVA was used to compare differences between groups that were split into genotype and conditions, and the Sidak's multiple comparison post-hoc test was used for pairwise comparisons.

Statistical Analysis

The statistical details of experiments can be found in figure legends. All statistical tests were performed in R (version 3.3.1). Graphs were generated in R using ggplot2 package, except for sleep profiles, which were generated in Pysolo.

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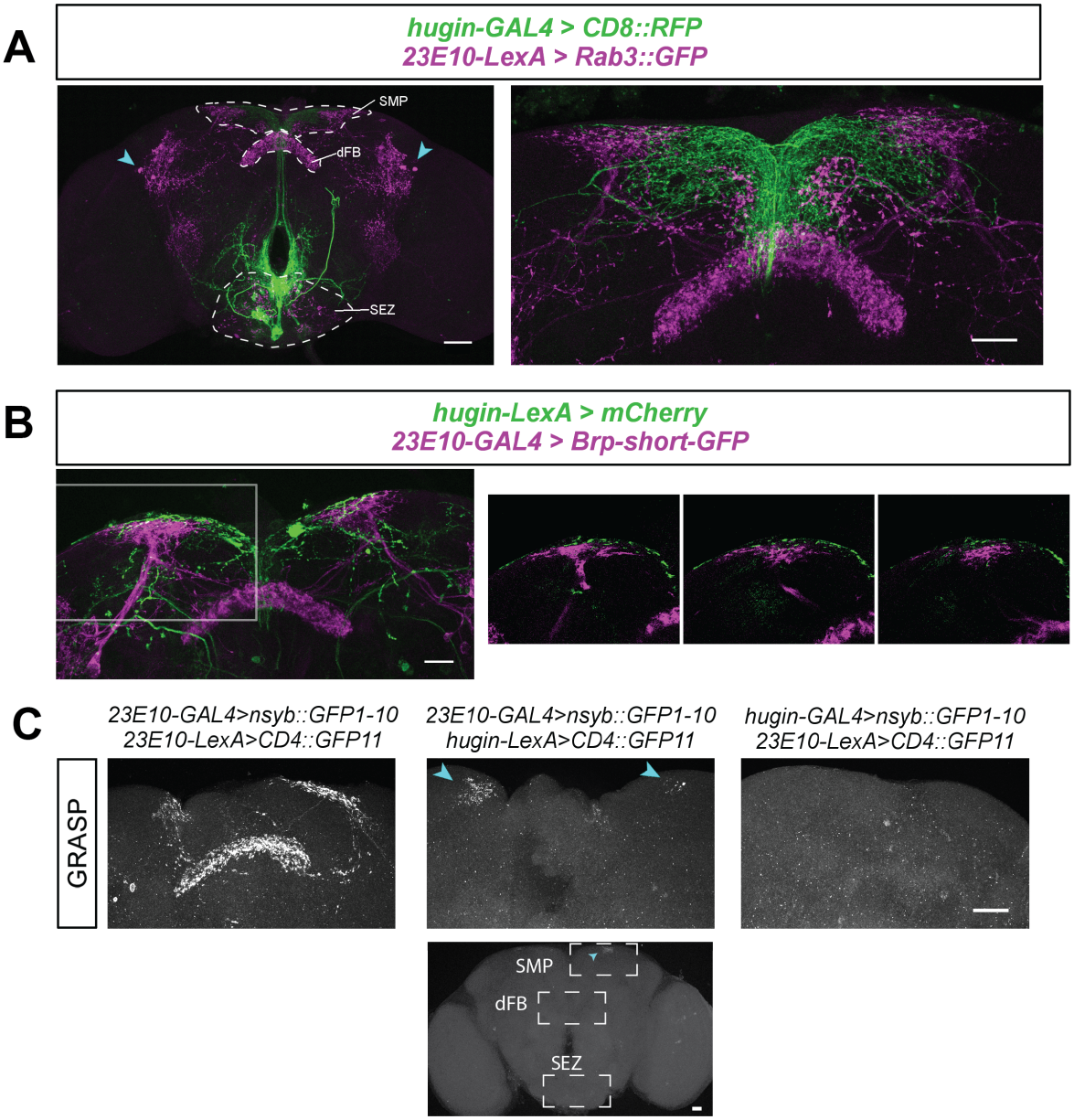


**Figure 1: Sleep-promoting dFB (dorsal fan-shaped body) neurons contact *hugin*+ circadian output neurons.**

**(A)** Co-labeling of *hugin*+ neurons with membrane marker (green) and *23E10*+ dFB neurons with RAB3::GFP, a presynaptic marker (magenta). The left image shows co-labeling of neurons in the whole fly brain; arrowheads indicate *23E10*+ cell bodies. Superior medial protocerebrum (SMP), dorsal fan-shaped body (dFB), and subesophageal zone (SEZ) regions are labeled. The right image shows the dorsal protocerebrum, where *hugin*+ projections intermingle with *23E10*+

projections in the SMP. **(B)** Co-labeling of *hugin*+ neurons with membrane marker (green) and *23E10*+ dFB neurons with BRP-short$^{GFP}$, a presynaptic marker (magenta). The left image shows co-labeling of neurons in the dorsal brain. The right series of images show single confocal sections of the region indicated by white box, where *hugin*+ projections intermingle with *23E10*+ projections in the SMP. **(C)** Synaptic nSyb::spGFP1-10 is expressed in presynaptic neurons and complementary spGFP11 expressed in putative postsynaptic neurons. GFP reconstitution occurs only if synaptic connectivity exists. C, Left: When both nSyb::spGFP1-10 and spGFP11 are expressed in *23E10*+ dFB neurons, GFP reconstitution occurs in the dFB and SMP. C, Middle, Top: Cyan arrowheads point to the GFP reconstitution in the SMP when nSyb::spGFP1-10 is expressed in *23E10*+ dFB neurons and spGFP11 is expressed in *hugin*+ neurons. C, Middle, bottom: GFP is only reconstituted in the SMP (not dFB or SEZ) when nSyb::spGFP1-10 is expressed in *23E10*+ dFB neurons and spGFP11 is expressed in *hugin*+ neurons. C, Right: No GFP reconstitution when nSyb::spGFP1-10 is expressed in *hugin*+ neurons and spGFP11 is expressed in *23E10*+ dFB neurons. Scale bars, A(left): 50 μm; A(right), B, C: 25 μm.

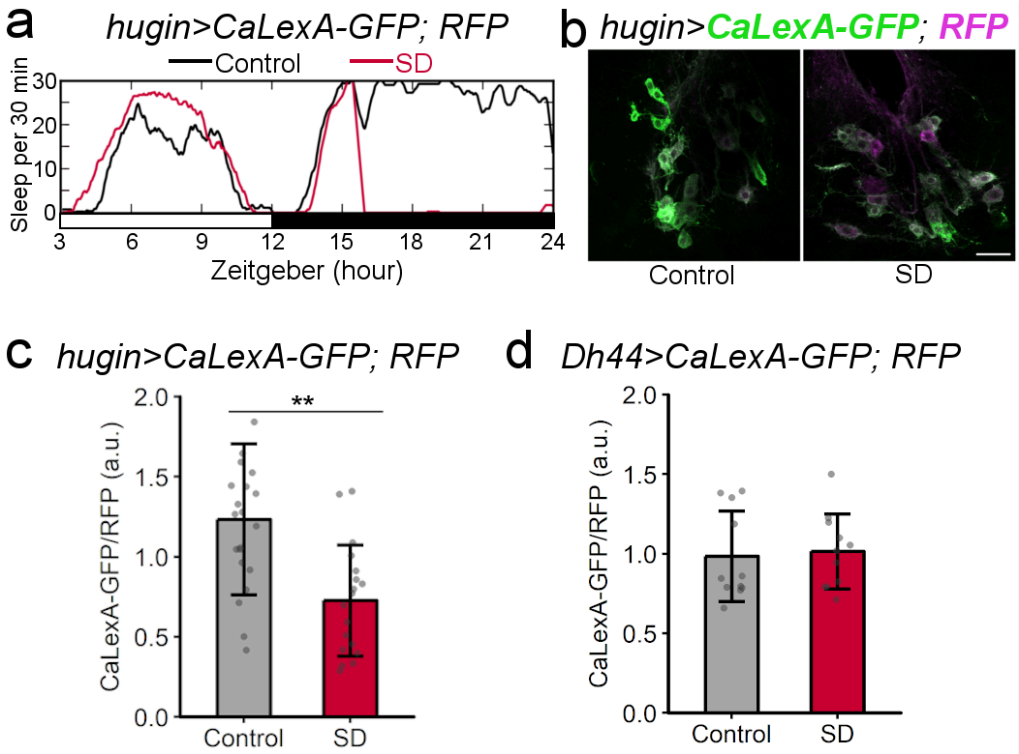


**Figure 2: $Ca^{2+}$ levels of *hugin*+ neurons are suppressed with sleep deprivation.**

**(A)** Sleep profiles of *hugin>CaLexA-GFP; RFP* flies subjected to no sleep deprivation (Control, black, n = 8 flies) or 9-hr sleep deprivation (SD, red, *n* = 8 flies). Sleep graphed as minutes per 30-minute bin over 21 hours. **(B)** Representative images show GFP reporting $Ca^{2+}$ levels via the CaLexA system and RFP normalizer signals in *hugin>CaLexA-GFP; RFP* flies from Control or SD groups. Max intensity projection images show *hugin*+ neurons in subesophageal zone. Scale bar, 25 µm. **(C)** Levels of GFP signal normalized to RFP signal in *hugin*+ cell bodies from Control (*n* = 21 flies) and SD (*n* = 18 flies) groups. **P = 0.000449, Welch's *t*-test. **(D)** Levels of GFP signal normalized to RFP signal in *Dh44*+ cell bodies from Control (*n* = 11 flies) and SD (*n* = 11 flies) groups. n.s., P = 0.782 by Welch's *t*-test.

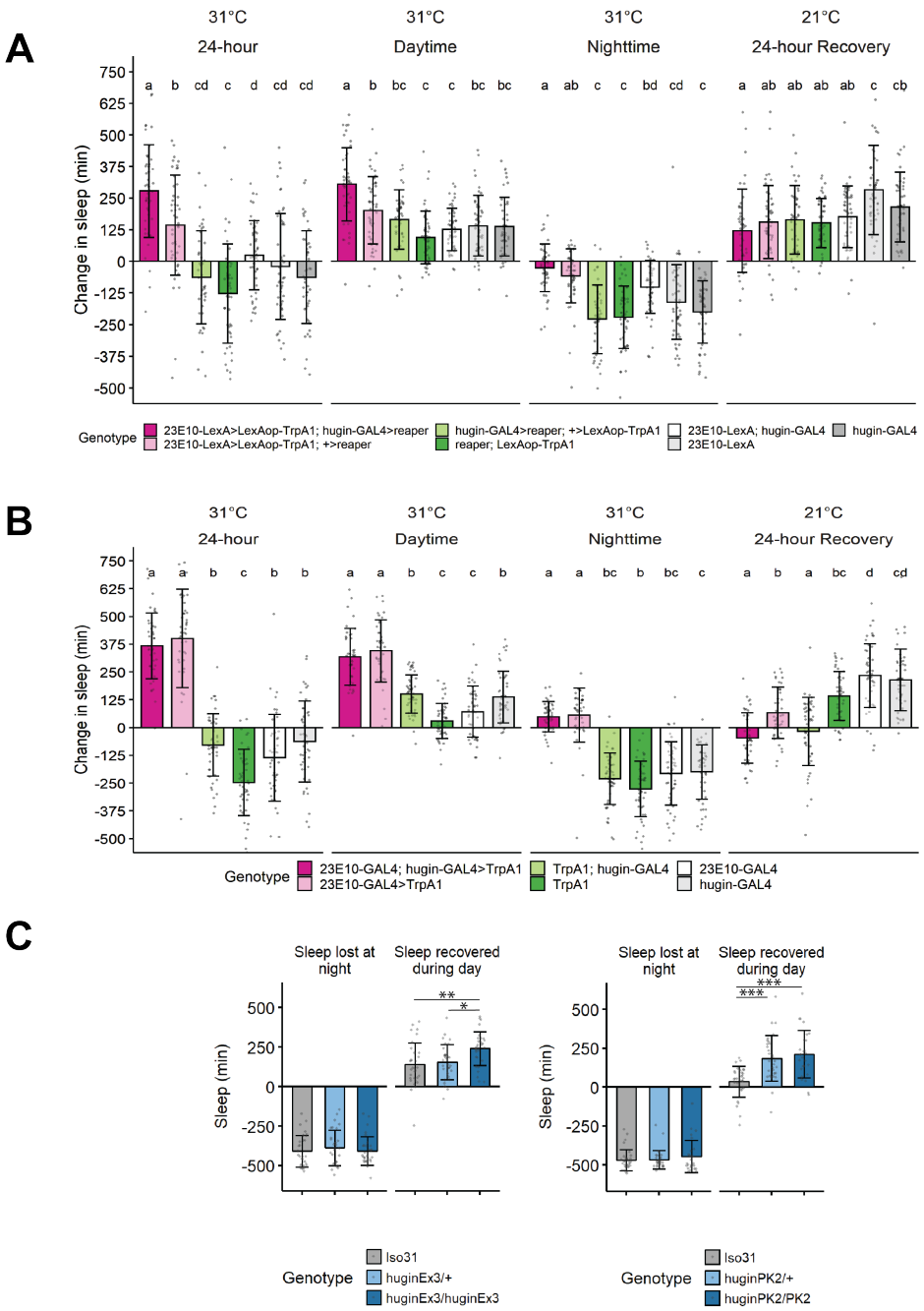


**Figure 3: *hugin+* neurons are effectors of *23E10+* sleep-promoting dFB neurons.**

**(A)** *23E10+* dFB neurons activated with *TrpA1* in flies where *hugin+* neurons were ablated using *reaper*. A, left 3 graphs: Changes in sleep amount with temperature dependent (31°C) activation of *23E10+* neurons, relative to sleep levels on the pre-activation day. Changes are shown for the 24h day and also split into day and night sleep. A, rightmost graph: Assay of recovery sleep

following *TrpA1* activation of *23E10*+ dFB neurons in the absence of *hugin*+ neurons. The higher temperature used to induce *TrpA1* decreases sleep in control flies, and so results in higher sleep the following day. Recovery sleep is plotted as the difference in 24h sleep on the day after heat-induced nighttime sleep loss relative to sleep amount on the pre-activation day. **(B)** Simultaneous activation of *23E10*+ dFB neurons and *hugin*+ neurons with *TrpA1*. B, left 3 graphs: Changes in sleep amount with temperature dependent (31$^{o}$C) activation of *23E10*+ neurons, relative to sleep levels on the pre-activation day. B, rightmost graph: Recovery sleep following *TrpA1* activation of *23E10*+ dFB neurons and *hugin*+ neurons. Recovery sleep is plotted as the difference in 24h sleep on the day after heat-induced nighttime sleep loss relative to sleep amount on the pre-activation day. **(C)** Hugin CRISPR mutants, $hugin^{\Delta Ex3}$ (left) and $hugin^{PK}$ (right), were mechanically sleep deprived from ZT15-24. Changes in night sleep amount between baseline and during the sleep deprivation and well as recovery sleep, change in sleep amount after sleep deprivation compared to baseline, are shown. For all panels: Circles are individual fly data points, and summary statistics are displayed as mean $\pm$ SD. Means compared with one-way ANOVA and Tukey's test. For panels A-C: Means sharing the same letter are not significantly different from each other (P > 0.05, Tukey's test). For panels D-E *P = 0.0147089, **P = 0.0030506, ***P = 0.0000160, 0.0000003 by Tukey's test.

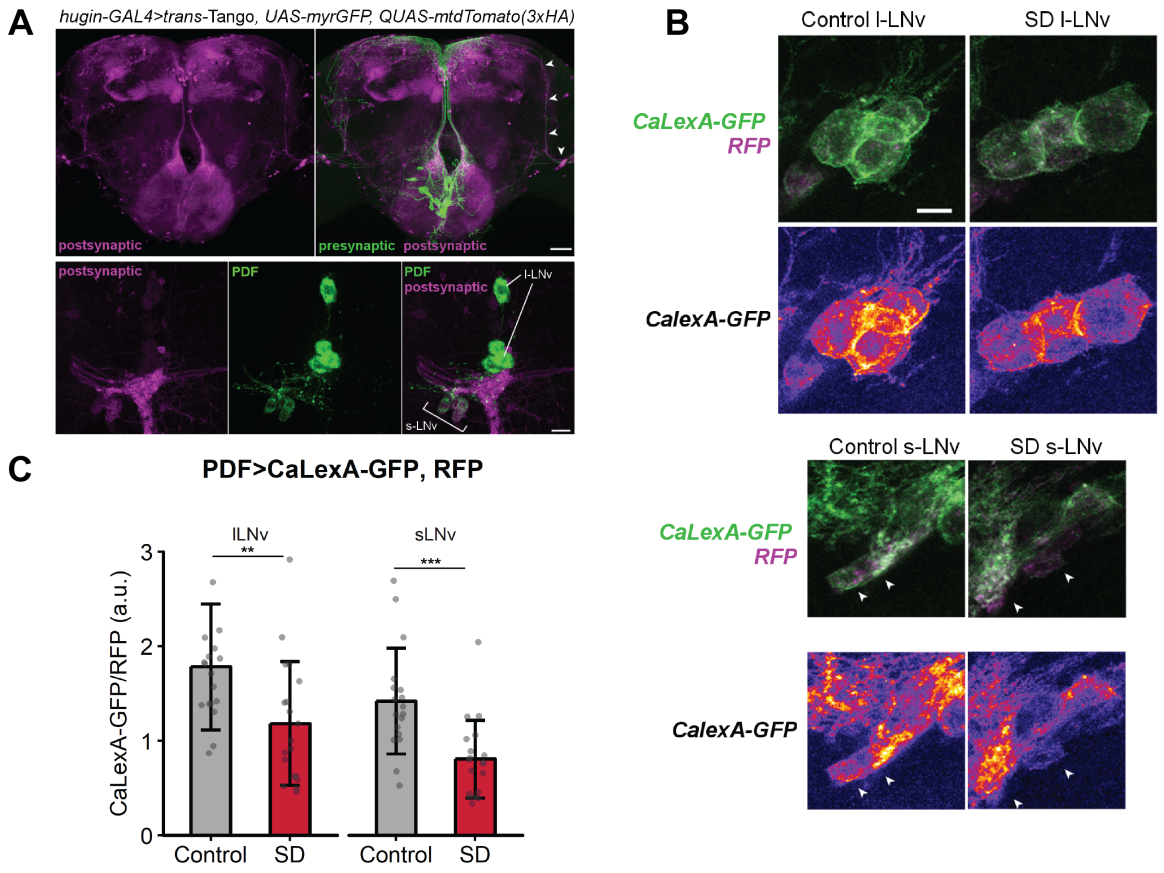


**Figure 4: *hugin*+ neurons target PDF-expressing clock neurons which show decreased $Ca^{2+}$ levels upon sleep deprivation.**

**(A)** *trans*-Tango ligand is expressed in *hugin*+ neurons (green). *trans*-Tango system reveals the synaptic partners (magenta) of *hugin*+ neurons in the brain. Panel A (top) image is a max intensity projection from the anterior side, and arrowheads indicates postsynaptic signal that resembles the projections of PDF+ neurons. A (bottom) Co-labeling of PDF peptide (green) and postsynaptic signal (magenta) in flies with *trans*-Tango ligand expressed in *hugin*+ neurons. PDF+ s-LNvs are postsynaptic to *hugin*+ neurons. s-LNv, small ventrolateral neurons, l-LNv, large ventrolateral neurons. Scale bars, A top: 50 μm; bottom: 15 μm. **(B)** Representative images of l-LNvs or s-LNvs from a *Pdf>CaLexA-GFP; RFP* fly in Control or SD group. Top row shows merged images of GFP signal reporting $Ca^{2+}$ levels with the CaLexA system and RFP normalizer signal. Bottom row shows “Fire” pseudocolor image of CaLexA-GFP signal

(blue/purple=low intensity and yellow/white=high intensity). Scale bar, 10 μm applies for all images in this panel. **(C)** *Pdf>CaLexA-GFP; RFP* flies were subjected to no sleep deprivation (Control, gray) or 9-hr sleep deprivation (SD, red). Graph shows GFP levels normalized to RFP levels in *Pdf*+ large ventrolateral neurons (l-LNv) or small ventrolateral neurons (s-LNv) from Control ($n$ = 18 flies) and SD ($n$ = 19 flies) groups. **P = 0.00910, ***P = 0.000655 by Welch's *t*-test.

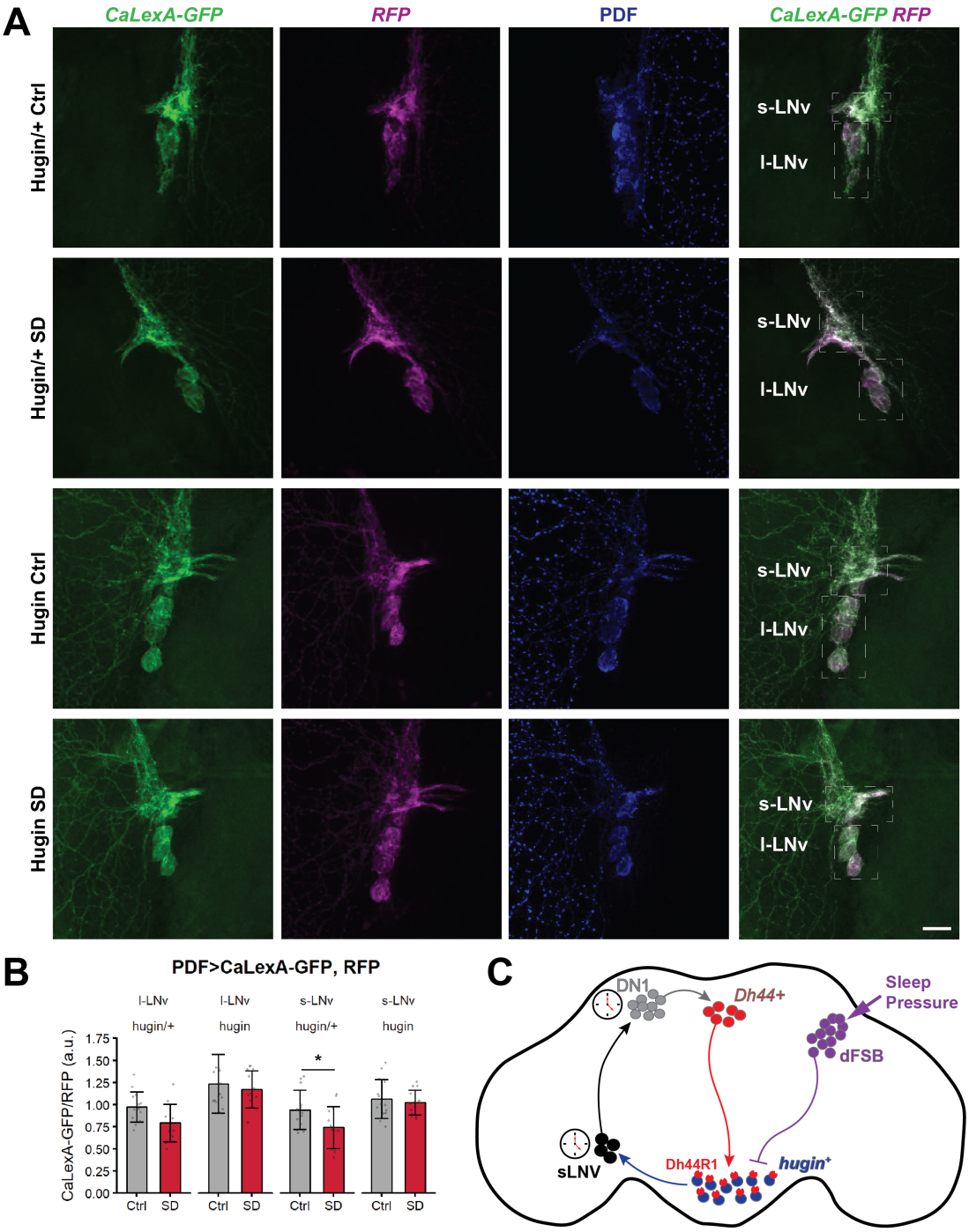


**Figure 5: Hugin peptide regulates the decrease in $Ca^{2+}$ levels of *Pdf*-expressing clock neurons after sleep deprivation.**

**(A)** Representative images of l-LNvs and s-LNvs from a *Pdf>CaLexA-GFP; RFP* flies in Control or SD group. The fourth column shows merged images of GFP signal reporting $Ca^{2+}$ levels with

the CaLexA system and RFP normalizer signal. Note the lower green signal in the *hugin*$^{\Delta Ex3/+}$ heterozygous fly subjected to SD (second row) compared to the *hugin*$^{\Delta Ex3/+}$ heterozygous control fly (top row). 25 µm scale bar applies for all images in this panel **(B)** $Ca^{2+}$ levels in *Pdf*+ neurons were measured with *Pdf>CaLexA-GFP; RFP* reporter in *hugin*$^{\Delta Ex3/+}$ heterozygous or *hugin*$^{\Delta Ex3/\Delta Ex3}$ mutant flies that were subjected to no sleep deprivation (Control, gray) or 9-hr sleep deprivation (SD, red). Graph shows GFP levels normalized to RFP levels in *Pdf*+ l-LNvs or s-LNvs (*n* = 13 or 14 flies per group). l-LNv: Two-way ANOVA (genotype x condition) revealed a significant main effect of genotype ($F(1, 47) = 23.29$, $P < 0.0001$), a nonsignificant effect of condition ($F(1,47) = 3.68$, $P = 0.061$), and a nonsignificant interaction between factors ($F(1, 47) = 0.604$, $P = 0.441$). s-LNv: Two-way ANOVA revealed a significant main effect of genotype ($F(1, 49) = 12.47$, $P = 0.0009$), a significant effect of condition ($F(1,49) = 4.36$, $P = 0.042$), and a nonsignificant interaction between factors ($F(1, 49) = 1.852$, $P=0.180$). * $P = 0.0385$, significantly different by Sidak's multiple comparison test. **(C)** Proposed model for regulation of a circadian output circuit by sleep homeostatic drive**.** *23E10*+ dorsal fan-shaped body (dFB) neurons are effectors of a sleep homeostatic circuit and promote sleep. During high sleep drive, the *23E10*+ dFB neurons (purple) promote sleep and dampen the output activities of the circadian system through inhibiting *hugin*+ circadian output neurons (blue) and sLNV clock neurons (black).